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Histone Methylation and Epigenetic Silencing in Breast Cancer

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14. ABSTRACT
This research is to investigate the role of a chromatin-modifying enzyme, called EZH2, in breast cancer epigenetics and to develop strategies to identify chemical inhibitors of this enzyme. EZH2 is a histone methyltransferase which modifies lysine-27 of histone H3, an epigenetic mark linked to gene silencing and implicated in tumor suppressor silencing during breast cancer progression. Progress on this project includes: 1) Identification of target genes directly silenced by EZH2 in breast cancer cells, 2) Mapping of EZH2 association within the chromatin of one such target gene, 3) Demonstration that a DNA-binding protein, called YY1, co-localizes at this target chromatin, and 4) Evidence that YY1 is needed for silencing and EZH2 chromatin binding at this target gene. These results suggest that YY1 binding sites may help define response elements that recruit and mediate EZH2 silencing in breast cancer cells. The definition of EZH2 response elements is required to engineer a breast cancer cell-based bioassay to screen for EZH2 inhibitors. These inhibitors provide important drug compounds to test as part of emerging epigenetic therapies to combat cancer.

15. SUBJECT TERMS
cancer epigenetics, histone modification, transcriptional repression, chromatin, histone methyltransferase, Polycomb group

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Introduction

This research project is to investigate a chromatin-modifying enzyme, called EZH2, which is implicated in epigenetic modifications that contribute to breast cancer progression (Bracken et al. 2003; Kleer et al. 2003; Raaphorst et al. 2003; Cha et al. 2005; Bachmann et al. 2006; Collett et al. 2006; Ding et al. 2006). EZH2 is a histone methyltransferase that modifies histone H3 on lysine-27 (Cao et al. 2002; Kuzmichev et al. 2002), which is a chromatin modification deployed in gene silencing (Cao and Zhang 2004). The purposes of this research are to: 1) reveal basic mechanisms and consequences of EZH2 function in breast cancer cells and 2) develop a breast cancer cell line-based bioassay to screen for inhibitors of the EZH2 histone methyltransferase. This research entails identification of individual target genes silenced by EZH2 in breast cancer cells, the mapping and characterization of DNA elements within these genes that mediate EZH2 silencing, and exploitation of these EZH2 response elements to engineer cell lines that enable identification of small molecule inhibitors of EZH2.

Body

The body of this Annual Progress Report is organized with respect to individual Tasks within the Statement of Work (SOW).

Task I-2A: To identify target genes that are silenced by EZH2 in breast cancer cell lines

As described in earlier Progress Reports, we have identified two target genes that are silenced by EZH2 in SKBR3 and MCF7 breast cancer cells. These EZH2-responsive genes are CCND2, which encodes the cell cycle regulatory protein cyclin D2, and MYT1, which encodes a transcription factor. We selected CCND2 for further detailed analysis, including chromatin immuneprecipitation (chromatin IP) studies to determine if and where EZH2 binds within the regulatory DNA region of this target gene. These studies revealed that EZH2 associates with the promoter region and with an upstream region located from -1.6 to -3.3 kb relative to the CCND2 transcription start site. These results implied that the CCND2 regulatory region contains response elements that attract EZH2 to this target gene and mediate EZH2 transcriptional silencing.

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which methylates target gene chromatin on histone H3-K27 (Cao et al. 2002; Kuzmichev et al. 2002). In order for PRC2-mediated silencing to occur at the majority of its known chromatin targets, it must cooperate with a second silencing complex, called PRC1. Indeed, we found that a PRC1 subunit, BMI-1, shows coincident distribution with the PRC2 subunit (EZH2) on CCND2 regulatory DNA. Taken together, these results substantiated CCND2 as an appropriate model target gene to study in breast cancer cells since it attracts both major Polycomb silencing complexes. In the most recent project period, we turned our attention to delimiting the DNA regions upstream of CCND2 that mediate EZH2 silencing.

Task II-1: To identify and test DNA fragments from EZH2 target genes that can mediate EZH2 silencing (EZH2 response elements)

Neither PRC2 nor PRC1 contain subunits that are sequence-specific DNA-binding proteins. Furthermore, little is known about the DNA elements in mammalian target genes that recruit PRC2 and PRC1. Thus, a major goal is to identify the response elements within the CCND2 target gene and the protein factors, referred to here as "recruiters", that bind these elements and serve to attract EZH2 and its associated silencing machinery.

Based on the work of others using embryonic stem cells and muscle cells, the YY1 and OCT4 DNA-binding proteins have been suggested as potential EZH2 recruiters (Caretti et al. 2004; Squazzo et al. 2006; Endoh et al. 2008). To address if these proteins might function as EZH2 recruiters in breast cancer cells, we performed chromatin IPs to map their associations with CCND2 regulatory DNA. As presented in an earlier Progress Report, we found that both YY1 and OCT4 bind to the same regions of CCND2 as EZH2: the promoter region and a more upstream region encompassing from -1600 to -3300 relative to the transcription start site. Thus, the CCND2 upstream region provided a starting point to search for and define EZH2 response elements, which may feature YY1 and/or OCT4 binding sites.

A major effort was then pursued to address the functional importance of YY1 and OCT4 in 1) silencing CCND2 expression and 2) recruiting EZH2 to the CCND2 target gene in breast cancer cells. As described in the previous annual report, technical issues prevented us from assessing the functional role of OCT4 in CCND2 silencing in SKBR3 breast cancer cells. However, we succeeded in analyzing the functional role of YY1. We showed that YY1 knockdown by RNA interference (RNAi) causes loss of EZH2 (a PRC2 subunit) and BMI-1 (a PRC1 subunit) from the CCND2 target gene and a concomitant robust desilencing of CCND2.

These results suggested that the YY1 DNA-binding protein recognizes and binds CCND2 regulatory DNA and that this, in turn, helps recruit EZH2 to the target locus. If this is correct, then YY1 binding sites should contribute functionally to the CCND2 DNA fragments that mediate EZH2 silencing (EZH2 response elements). Indeed, we found that there are 3 predicted YY1 sites in the CCND2 promoter region (spanning +250 to -450) and another 6 YY1 sites in the CCND2 upstream region (encompassing -1600 to -3300). The locations of these predicted YY1 sites are represented by asterisks in Figure 1A. Similar results implicating YY1 in Polycomb silencing of a *Hox* target gene in differentiating human ES cells have recently been obtained (Woo et al. 2010).

To physically define EZH2 response elements, and assess the contributions of particular YY1 binding sites, we required a functional assay for EZH2 silencing in breast cancer cells. Towards this end, we generated a set of luciferase reporter constructs (Fig. 1B) for use in transient transfection assays. These constructs contain portions of CCND2 regulatory DNA, encompassing promoter and/or upstream regions, that our chromatin IP assays showed are associated with YY1, EZH2, and BMI-1. Our initial approach was to co-transfect each of these reporters along with an EZH2 expression construct to test for reporter silencing due to increased EZH2 levels. This approach employed MCF10A mammary cells since endogenous levels of EZH2 are lower in MCF10A cells than in SKBR3 cells. However, this over-expression/reporter assay proved to be insufficiently robust. Specifically, only one construct out of five tested, which combines the CCND2 promoter with an SV40 enhancer (#2 in Fig. 1B), yielded reproducible silencing via this EZH2 over-expression approach. Furthermore, Western blots (not shown) demonstrated that the increase in EZH2 levels after co-transfection into MCF10A cells was limited to two-fold or less. We concluded that this modest effect was limiting the usefulness of this assay for defining EZH2-responsive fragments.

Consequently, we pursued implementation of an alternative reporter assay which relies upon EZH2 knockdown rather than over-expression. We reasoned that that this knockdown approach was more likely to yield a robust assay for EZH2-mediated silencing in breast cancer cells because: 1) we had already established RNAi conditions that routinely produce 8- to 10-fold EZH2 depletion in SKBR3 cells and 2) a similar reporter transfection/knockdown assay was used successfully to demonstrate reporter silencing by a *Hox* gene DNA fragment targeted by PRC2 and PRC1 in mesenchymal stem cells (Woo et al. 2010). We transfected SKBR3 cells with the aforementioned reporter constructs (Fig. 1B), subjected them to RNAi

treatment to deplete EZH2, and then assayed for increased luciferase levels as a readout for desilencing. Parallel transfected samples were treated with either non-targeted control (NT2) dsRNAs or EZH2 dsRNAs and luciferase levels were compared. As shown in Fig. 1C, reporter constructs bearing either the CCND2 promoter (upper left panel), a CCND2 upstream fragment (upper right panel), or a CCND2 fragment encompassing both promoter and upstream regions (lower left panel) show significant desilencing in response to EZH2 knockdown. A control reporter construct that lacks CCND2 regulatory DNA and therefore should not be responsive to EZH2 levels was tested in parallel. As expected, and in contrast to the CCND2 constructs, this SV40 enhancer plus SV40 promoter construct showed no change in luciferase expression upon EZH2 depletion (Fig. 1C, lower right panel). We conclude that this transfection/ knockdown assay succeeds at providing a functional readout to identify CCND2 DNA elements that mediate EZH2 silencing.

Having established a functional reporter assay, we next extended the analysis to determine if the same CCND2 fragments that respond to EZH2 loss also show desilencing upon knockdown of YY1. Our previous chromatin IP results, showing that YY1 loss leads to EZH2 dissociation from CCND2 chromatin, suggests that loss of either repressor should trigger CCND2 reporter desilencing. Indeed, we found that RNAi treatments to deplete YY1 led to marked increases in luciferase expression from the CCND2 constructs but not from the control construct (Fig. 2). Taken together, these results indicate that CCND2 fragments bearing YY1 binding sites can mediate gene silencing that depends on YY1 and EZH2.

To complete this study, we now plan to delimit and better define the EZH2 response elements within the CCND2 upstream region. There are very few examples of defined EZH2 response elements (also called Polycomb response elements, PREs) within mammalian genes (Sing et al. 2009; Woo et al. 2010) and, to our knowledge, none have yet been demonstrated to function in breast cancer cells. To delimit locations of the response elements, we will use the reporter assay to test smaller CCND2 upstream fragments for YY1- and EZH2-mediated silencing. We have generated trimmed-down CCND2 constructs (Fig. 3, constructs 2 and 3) to test next in the reporter desilencing assay. We will then use whichever trimmed-down construct that maintains robust desilencing to serve as a platform for testing site-directed mutations that surgically disrupt the predicted YY1 sites. The primary goal is to determine if individual YY1 sites comprise key functional modules within this mammalian EZH2 response element. Accomplishing this set of tests will yield two outcomes: 1) These tests will assess the intrinsic role of YY1 sites in recruiting EZH2 to target genes for silencing in breast cancer cells

and 2) The tests will identify a sufficiently delimited CCND2 fragment that can be used to engineer a breast cancer cell line bearing an integrated EZH2-responsive luciferase reporter. Such a cell line supplies a potential tool to deploy in high-throughout screens for EZH2 inhibitors, as originally envisioned in Task II-3.

Key Research Accomplishments

1) Identification of target genes that are directly silenced by EZH2 in breast cancer cells

2) Mapping of EZH2, BMI-1, YY1, and OCT4 sites of association within the CCND2 target gene in breast cancer cells

3) Demonstration that the transcription factor YY1 is required to recruit EZH2 to CCND2 target gene chromatin and for transcriptional silencing of CCND2 in breast cancer cells

4) Establishment of a transfection/knockdown assay to test CCND2 regulatory DNA fragments for function as EZH2 response elements

Reportable Outcomes

1) Review Article: Simon, J.A and Lange, C.A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. ***Mutation Research*** 647, 21-29. In special issue on "Epigenetics of development and human disease."
(listed in previous Annual Progress Report).

Conclusion

This research has identified individual EZH2 target genes in breast cancer cells. One of these target genes, CCND2, is now being exploited in further studies to identify and delimit DNA elements responsible for gene silencing by EZH2. This research has also provided

insight to mechanisms that recruit EZH2 to target genes in breast cancer cells. Specifically, the zinc finger DNA-binding protein, YY1, is implicated in targeting EZH2 to the CCND2 target gene. The identification, characterization, and harnessing of these EZH2 response elements provide a key component in cell-based strategies for isolating small molecule inhibitors of the EZH2 chromatin-modifying enzyme. Such inhibitors provide important lead compounds for the development and optimization of potential therapeutics that block EZH2 function. These inhibitors, and their derivatives, may find use in emerging strategies to combat cancer progression via drugs that alter epigenetic states of genomes in cancer cells (Egger et al. 2004; Lyko and Brown 2005; Yoo and Jones 2006).

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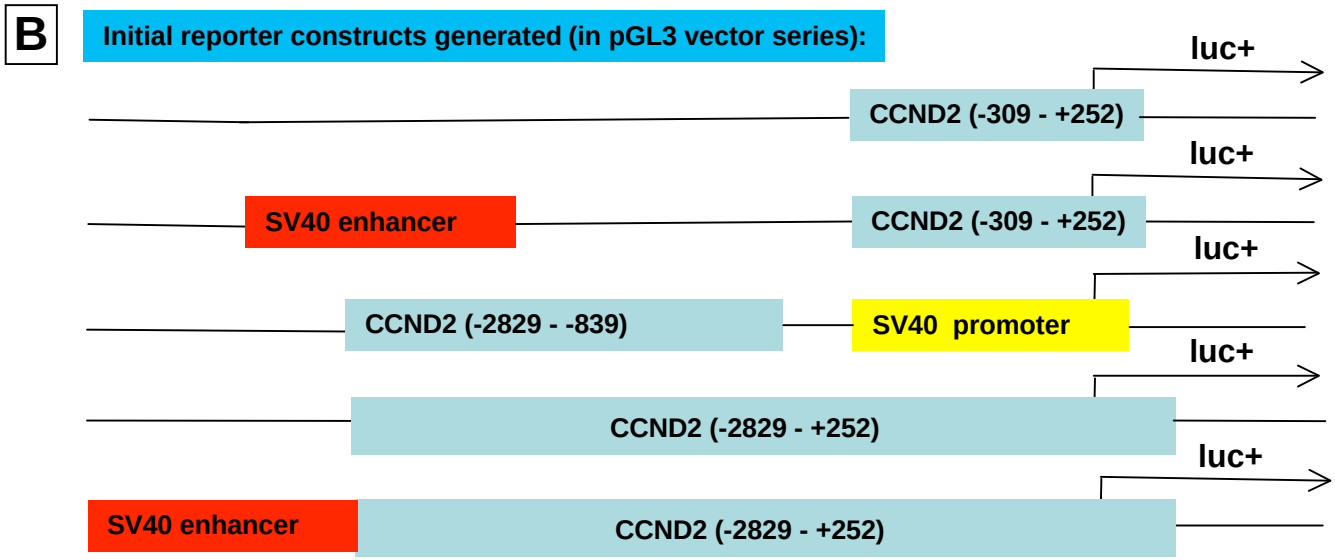
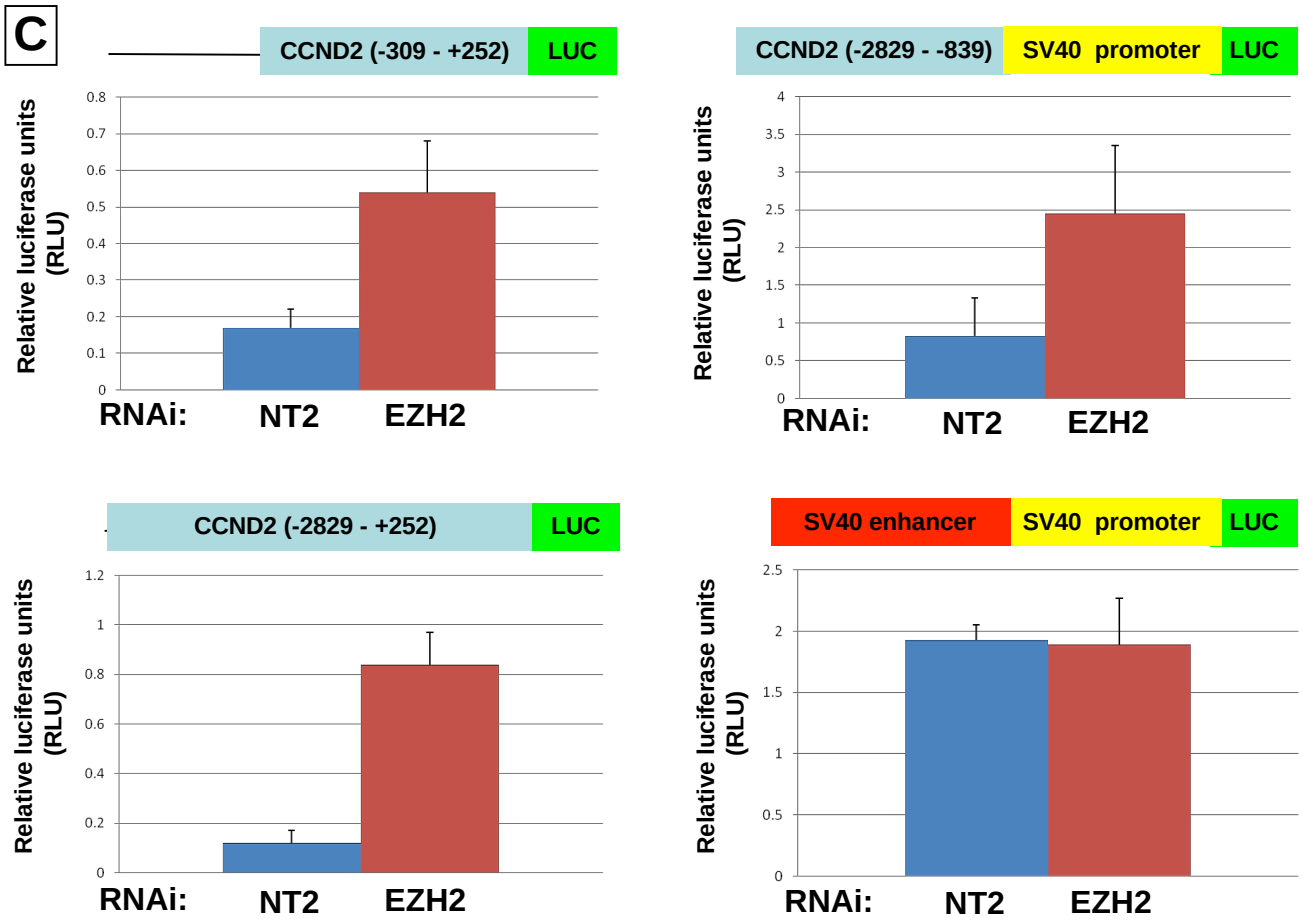
Supporting Data

Figures 1-3, which accompany the Body of the Progress Report, are appended below.

Figure 1





(For Fig.1 Legend, please see next page.)

Figure 1. (previous page)
Reporter constructs and assays for EZH2 silencing in breast cancer cells.
A) Map of the CCND2 promoter and upstream region encompassing DNA from -3600 to +250 bp relative to the transcription start site (curved arrow). Asterisks represent locations of predicted YY1 binding sites based on matches to consensus sequence. B) All constructs contain indicated regulatory DNA fused to the coding region of the firefly luciferase gene. Some constructs contain intact segments of CCND2 upstream DNA whereas others are composites of CCND2 DNA with the SV40 enhancer or promoter. C) Bar graphs depict reporter assay results. Blue bars show relative luciferase expression levels (firefly versus *Renilla* control) in SKBR3 cells treated with a control non-targeted dsRNA (NT2) and red bars depict relative luciferase levels in cells treated with EZH2 dsRNA. Transfection constructs used in each assay are displayed above each bar graph. Reporter desilencing is observed upon EZH2 depletion with all constructs except a control construct lacking CCND2 DNA (lower right panel). Error bars represent standard deviation derived from assays performed in triplicate.

Figure 2

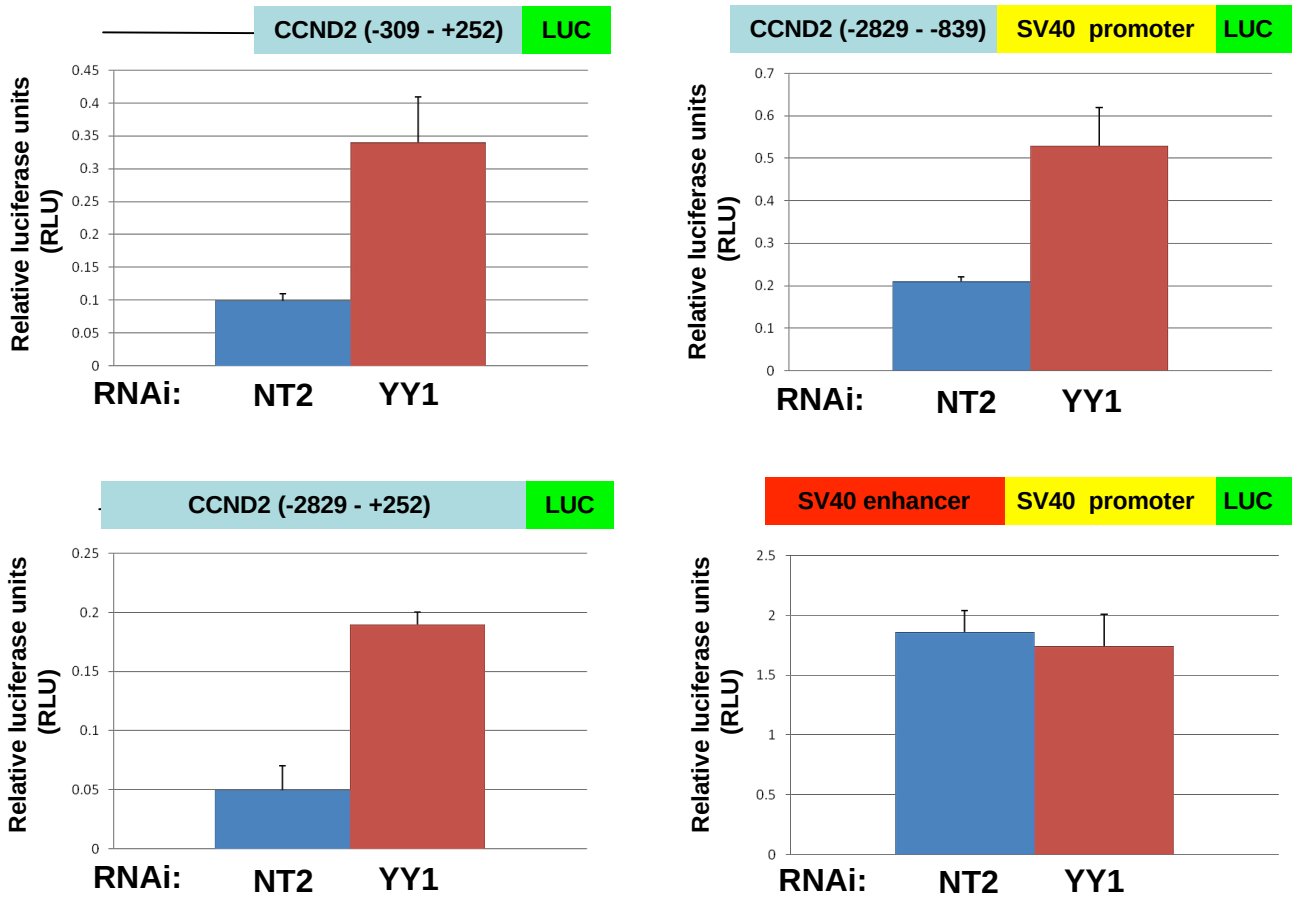


Figure 2. Assays for YY1-mediated silencing in breast cancer cells.
Bar graphs depict reporter assay results. Blue bars show relative luciferase expression levels (firefly versus *Renilla* control) in SKBR3 cells treated with a control non-targeted dsRNA (NT2) and red bars depict relative luciferase levels in cells treated with YY1 dsRNA. Transfection constructs used in each assay are displayed above each bar graph. Reporter desilencing is observed upon YY1 depletion with all constructs except a control construct lacking CCND2 DNA (lower right panel). Error bars represent standard deviation derived from assays performed in triplicate.

Figure 3

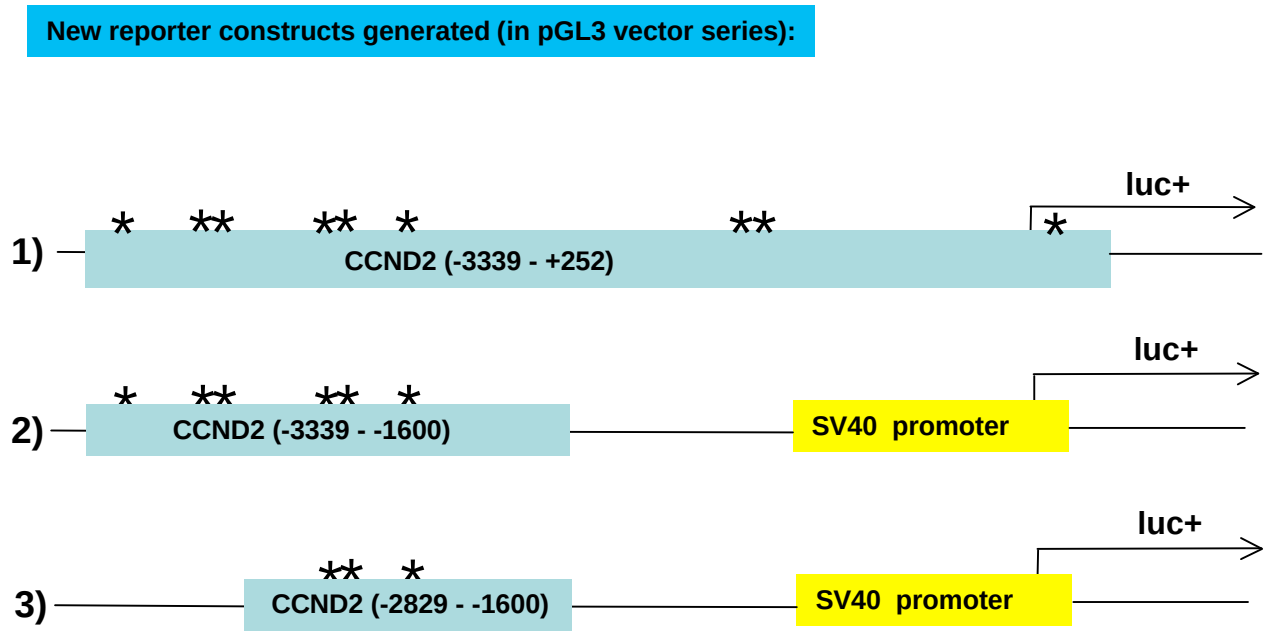


Figure 3. Reporter constructs to delimit EZH2 response element in CCND2 upstream DNA. Constructs contain indicated regulatory DNA fused to the coding region of the firefly luciferase gene. The first construct contains an intact segment of CCND2 upstream DNA whereas the other two are composites of CCND2 DNA with the SV40 promoter. Asterisks represent locations of predicted YY1 binding sites based on matches to consensus sequence. Construct #2 contains a cluster of 6 YY1 sites within CCND2 upstream DNA and construct #3 contains 3 of these sites.